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(57) Abstract <p>The present invention provides a synthetic FIV polypeptide comprising an amino acid sequence substantially corresponding to all or a portion of the FIV envelope protein, or an antigenic fragment or functionally-equivalent variant thereof, in which the proteolytic cleavage site between the transmembrane and surface polypeptides of the native FIV envelope precursor protein has been eliminated and which comprises at least a portion of a transmembrane polypeptide and at least a portion of a surface polypeptide. Also provided are nucleic acid molecules encoding such polypeptides, methods for their recombinant expression, vaccine compositions containing them, and their use in combatting FIV.</p>		

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ANTI-FELINE IMMUNODEFICIENCY VIRUS (FIV) VACCINES

The present invention relates to feline immunodeficiency virus (FIV) and to the development of vaccines for use in protecting cats and kittens against FIV infection.

FIV is a recently discovered T-lymphotropic lentivirus which infects cats to produce an AIDS-like syndrome. FIV, while exhibiting morphological and pathological similarity, has however been shown to be antigenically distinct from the human immunodeficiency virus (HIV) (Pederson et al., Science 235: 790-793, 1987). Infected cats and kittens show a generally debilitating AIDS-like disease with intermittent symptoms (eg. lymphadenopathy, leucopenia and anaemia), characterised by a severe impairment of immune function as a result of loss of CD⁴⁺ T cells, resulting in susceptibility to secondary opportunistic infection and leading ultimately to death.

Epidemiological studies have shown FIV infection to be widespread worldwide and the disease is rapidly acquiring significant clinical importance from a veterinary point of view. Efforts have accordingly recently begun to be directed to the development of vaccines against FIV but whilst preliminary results with vaccines based on whole infected cells or whole virus have proved promising, (Yamamoto et al., AIDS Research and human retroviruses 7: 911-922, 1991), there are as yet no reports in the literature of successful immunisation of cats against FIV infection using a sub-unit or peptide-based vaccine. No commercial vaccines are currently available.

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Despite its ubiquitous presence, FIV does not appear to be capable of cat to human transmission. Nonetheless, FIV shares with HIV and other mammalian lentiviruses similarities in genome organisation, biological properties, the propensity for persistent infection in the natural host, with its concomitant pathological manifestations (eg. decline in CD4+ lymphocytes, both in vivo and in vitro, gradual loss of immune function, and opportunistic infection). Thus the development of experimental models of non-human lentivirus infections, such as FIV, may facilitate the design of vaccine and therapeutic strategies for HIV infection of humans.

The molecular structure of FIV, in terms of genome organisation and nucleotide sequence, has been studied (Talbot et al., PNAS, 86: 5743-5747, 1989; Olmstead et al., PNAS 86: 8088-8092, 1989) but as yet progress towards identifying regions of particular immunogenic importance has been slow and most proposals currently under investigation involve whole undisrupted virus.

There is therefore a continuing need for an effective candidate vaccine against FIV, particularly for a sub-unit or peptide-based vaccine, both for combatting FIV infection in cats and kittens and to serve as a useful animal model for HIV infection in man in which experimental vaccines may be evaluated. The present invention is directed towards providing such a candidate vaccine.

In particular, we have directed our efforts towards the envelope glycoprotein of FIV, which we believe may be important for virus infectivity and a major target, both for cytotoxic T cells and neutralising antibodies, in the host immune response to FIV infection.

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While preliminary investigations with whole undisrupted FIV virus-based vaccines proved promising, initial studies with disrupted viruses or with the FIV envelope protein alone were disappointing and acceptable levels of protective immunity obtained with FIV envelope protein immunisation could not be demonstrated. We believe that the problem may be due, at least in part, to shedding of the envelope glycoprotein from the surface of the purified virions.

The envelope glycoprotein of FIV is expressed initially as an 856 amino acid precursor protein having an estimated molecular weight of between about 130 kd and 160 kd which undergoes endoproteolytic cleavage to yield a mature complex, comprising in non-covalent association, an integral transmembrane glycoprotein (TM) of approximately 36-43 kd molecular weight and a peripheral outer membrane surface glycoprotein (SU) of estimated molecular weight 100-120 kd (Miyazawa et al., J. Virol., 65: 1572-1577, 1991). The association between the TM and SU proteins is weak and liable to breakage, for example in viral protein purification procedures. We now propose, according to the present invention, a recombinant full-length FIV envelope precursor protein in which the cleavage site between the TM and SU polypeptides has been eliminated, either by deletion or modification, such that cleavage can no longer occur.

One aspect of the present invention accordingly provides a synthetic FIV polypeptide comprising an amino acid sequence substantially corresponding to all or a portion of the FIV envelope protein, or an antigenic fragment or functionally-equivalent variant thereof, in which the proteolytic cleavage site between the transmembrane and surface polypeptides of the native FIV envelope precursor protein has been eliminated and which

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comprises at least a portion of a transmembrane polypeptide and at least a portion of a surface polypeptide.

A further aspect of the invention provides such a synthetic FIV polypeptides and antigenic fragments and functionally-equivalent variants thereof for use in combatting FIV infection in animals, eg. mammals by stimulating an immune response against FIV.

Alternatively viewed, the invention can also be seen to provide the use of such synthetic FIV polypeptides, and antigenic fragments and functionally equivalent variants thereof according to the present invention, in the preparation of a composition for combatting FIV infection in animals, eg. mammals, preferably for stimulating an immune response against FIV.

The term "polypeptide" as used herein defines both long chain polypeptides and shorter peptide sequences.

As mentioned above, included within the scope of invention are functionally-equivalent variants and fragments of synthetic FIV polypeptide according to the invention. "Functionally equivalent" as used above in relation to the polypeptide amino acid sequences defines polypeptides related to or derived from the native FIV envelope protein where the amino acid sequence has been modified by single or multiple amino acid substitution, addition or deletion, and also sequences where the amino acids have been chemically modified, including by glycosylation or deglycosylation, but which nonetheless retain immunogenic or other FIV-combatting activity eg. are capable of raising neutralising antibodies and/or functional immunity in the host. Such functionally-equivalent variants may occur as natural biological variations or may be prepared using known techniques,

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for example functionally equivalent recombinant polypeptides may be prepared using the known techniques of site-directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids.

Modification of the amino acid sequences to obtain functionally-equivalent variant sequences may be by amino acid substitution, as long as the immunogenicity of the polypeptide is not affected. Thus for example, an amino acid may be replaced by another which preserves the physicochemical character of the polypeptide or its epitope(s) eg. in terms of charge density, hydrophilicity/hydrophobicity, size and configuration and hence preserve the immunological structure. For example A may be replaced by G or vice versa, V by A, L or G; K by R; S by T or vice versa; E by D or vice versa; and Q by N or vice versa. Generally, the substituting amino acid has similar properties eg. hydrophobicity, hydrophilicity, electronegativity, bulky side chains etc. to the amino acid being replaced.

"Addition" variants include amino and/or carboxyl terminal fusions, for example by addition of amino acid sequences of up to 300 eg. up to 200 or 100 residues, as

well as intrasequence insertions of single or multiple amino acids. Amino acid sequences added may be those provided in the corresponding positions in the FIV envelope protein of other variants or other amino acids, eg. the whole or parts of other polypeptides or proteins. Longer peptides may comprise multiple copies of one or more of the polypeptide sequences.

Alternatively, multiple copies of the polypeptides (e.g. 5 to 20, preferably 8 to 15) may be coupled to a polyamino acid backbone, eg. a polylysine backbone to form so-called multiple antigen peptides (MAPs) as described for example by Tam in PNAS 85: 5400-5413,

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1988).

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Preferably, deletions or insertions are made in adjacent pairs eg. a deletion of two residues or insertion of two molecules. In all cases the proviso is that the modification preserves the immunogenicity of the polypeptide.

Exemplary functionally-equivalent variant polypeptides may thus include those displaying at least 50%, eg. at least 60 or 70%, or more preferably greater than 80% amino acid sequence homology. It should be noted however that functionally-equivalent variants, may exhibit overall sequence homology below the given percentages, but still fall within the scope of the present invention where they have conserved regions of homology.

It may in certain cases be convenient to include, where one does not occur naturally, one or more cysteine residues at the termini of the polypeptides, for example to enable specific carrier linkage or to permit disulphide bonding - this may be desirable to enable the polypeptides to mimic antigenic loops such as may appear on the surface of proteins and thereby enhance their immunogenicity.

A fatty acid or hydrophobic tail may also be added to the peptides to enhance immunogenicity and facilitate incorporation into delivery vehicles such as liposomes, novosomes and ISCOMS (Reid, Vaccine 10: 597-601, 1992).

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The amino acid residues of the synthetic polypeptides of the invention may be chemically modified, particularly at the ends of the molecule, and may take the form, for example, of amino acid ester or amides. Thus, for example, N- or C-terminal residues, eg. N- or C-terminal, particularly C-terminal cysteine residues may be chemically blocked or protected for example by an acetamido or other protecting group. A wide range of blocking groups are known in the art and may be used, including for example sulphonate, carboxymethyl, carboxamidomethyl, amino ethyl and similar groups.

The polypeptide may be linked to a carrier in order to create a conjugate which is immunogenically active. Any appropriate physiologically acceptable carrier may be employed, for example, a protein such as bovine serum albumin, thyroglobulin, ovalbumin or keyhole limpet hemocyanin. Recently, the concept of presenting multiple copies of viral peptides on the surface of particulate structures in a manner that resembles a virion has received much attention. This can be achieved either by producing virus/peptide chimaeras, eg. with poliovirus, or by linking the peptide to proteins which naturally self assemble into particles such as hepatitis B surface antigen, the Ty protein from

yeast, or the core protein from hepatitis virus (HBcAg). The polypeptide may also be linked to other FIV proteins or polypeptides thereby providing both an immunogen and a multivalent vaccine at the same time.

Rather than link a carrier sequence to a peptide in this way, a polypeptide may be prepared which itself incorporates an appropriate carrier sequence ie. as a fusion protein comprising the synthetic polypeptide(s) of the invention or a longer sequence incorporating such a polypeptide linked to a heterologous carrier sequence,

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as will be described in more detail below. Such carrier proteins are generally chosen so that the resultant product is physiologically acceptable.

Other modifications which may be made to enhance immunogenicity of the synthetic polypeptides include the introduction of, or coupling to longer amino acid sequences containing FIV helper T-cell epitopes. Particular mention may be made in this regard of particulate carrier proteins such as HBcAg which contain helper T-cell epitopes and thus have the dual advantages of a highly immunogenic mode of antigen presentation and the presence of helper T-cell epitopes. Such modifications, and many others which may enhance antigen presentation and/or delivery to the immune system, including those described above are well known in the art and are discussed and reviewed by Francis in *Vaccines*, Eds. Gregoriadis et al, Plenum Press, New York, p 13-23, 1991; *Sci. Progress* 74: 115-130, 1990 and by Francis & Clarke in *Meth. Enzymol.*, 178: 659-676, 1989.

The synthetic polypeptides of the invention may be presented as pharmaceutically or physiologically acceptable salts eg. acid addition salts. This may include both organic and inorganic salts such as those prepared for example from acids such as hydrochloric, hydrofluoric, sulfuric, sulfonic, tartaric, fumaric, hydrobromic, glycolic, citric, maleic, phosphoric, succinic, acetic, nitric, benzoic, ascorbic, p-toluenesulfonic, benzene-sulfonic, naphthalenesulfonic, propionic, and the like. Preferably, the acid addition salts are those prepared from hydrochloric acid, acetic acid, or succinic acid. Such salts may be prepared by conventional methods well known to those skilled in the art.

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Alternatively the peptide may be converted into a carboxylic acid salt, such as an ammonium or alkali metal salt eg. a sodium, potassium, or lithium salt etc.

As mentioned above, the synthetic FIV polypeptides according to the invention may be used to combat FIV infection in animals, and preferably to stimulate a host immune response against FIV. Such an immune response may comprise elements of both humoral and/or cell-mediated immunity to protect the host from FIV infection and/or kill or inhibit the virus, and may thus for example include the generation of immune effector molecules, antibodies or cells which damage, inhibit or kill the virus. Commonly, such a host-protective immune response may be manifested by the generation of antibodies which are able to neutralise the virus.

One of the ways in which the synthetic FIV polypeptides of the invention may exert their host protective effects is by raising neutralising antibodies which inhibit the growth and/or maintenance of the virus. Such neutralising antibodies, which may be mono- or polyclonal, form a further aspect of the invention as do vaccine compositions containing them and their use in the preparation of vaccine compositions for passively immunising hosts against FIV infection. Techniques for obtaining mono- or polyclonal antibodies are well known in the art.

FIV polypeptides according to the invention conveniently may be prepared by recombinant DNA technology using standard techniques such as those described for example by Sambrook et al., 1989, (Molecular Cloning, a Laboratory Manual, 2nd Edition, Cold Spring Harbour Press).

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A further aspect of the present invention thus provides a nucleic acid molecule comprising a nucleotide sequence substantially corresponding to all or a portion of the FIV env gene encoding the FIV envelope protein, or a sequence which is degenerate or substantially homologous with or which hybridises with any such aforesaid sequence, wherein in said nucleotide sequence the sequence encoding the proteolytic cleavage site between the transmembrane and surface polypeptides of the native FIV envelope protein has been eliminated, and wherein said nucleotide sequence encodes at least a portion of a transmembrane polypeptide and at least a portion of a surface polypeptide.

Additional aspects of the invention include such nucleic acid molecules for use in preparing FIV-combating compositions eg. vaccine compositions for stimulating an immune response against FIV in animals, and the use of such nucleic acid molecules according to the invention in the preparation of FIV-combating compositions eg. vaccine compositions for stimulating an immune response against FIV in animals.

Nucleic acid molecules according to the invention may be single or double stranded DNA, cDNA or RNA, preferably DNA.

Variations in the env coding region may occur between different isolates or serotypes, and strains of FIV of different geographical origin or even between different isolates from the same host and such variations which express as products capable of combating FIV eg. by stimulating an immune response against FIV, are included in the scope of this invention. Different FIV isolates and sequence divergence between them are described for example by Rigby et al., in Journal of General Virology (1993), 74, 425-436.

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"Substantially homologous" as used herein includes those sequences having a sequence homology of approximately 60% or more, eg. 70% or 80% or more, and also functionally-equivalent allelic variants and related sequences modified by single or multiple base substitution, addition and/or deletion. By "functionally equivalent" is meant nucleotide sequences which encode immunoreactive or immunogenic polypeptides eg. polypeptides which are capable of eliciting antibodies eg. neutralising antibodies or functional immunity in the host, or which otherwise are capable of combatting the FIV virus.

Nucleotide sequences as defined above which hybridise with regions of the FIV env gene, or with any degenerate, substantially homologous or functionally-equivalent sequence as defined above are also included within the scope of invention.

"Hybridisation" as used herein defines those sequences binding under non-stringent conditions (eg. 6 x SSC 50% formamide at room temperature) and washed under conditions of low stringency (eg. 2 x SSC, room temperature, more preferably 2 x SSC, 42°C) or conditions of higher stringency (eg. 2 x SSC, 65°C) (where SSC = 0.15M NaCl 0.015M sodium citrate, pH 7.2). Generally speaking, sequences which hybridise under conditions of high stringency are included within the scope of this invention, as are sequences which, but for the degeneracy of the code, would hybridise under high stringency conditions.

Methods for producing such derivative related sequences, for example by site-directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids are well known in the art, as are methods for determining whether the thus-modified nucleotide

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sequence has significant homology to the subject sequence, for example by hybridisation.

Provision of a nucleotide sequence according to the invention thus enables the recombinant FIV polypeptides to be obtained in significant quantities, thereby facilitating the development of anti-FIV vaccines and therapies.

Elimination of the proteolytic cleavage site in the nucleic acid molecules of the invention may conveniently be achieved by deletion or modification of the appropriate sequence coding for the cleavage site, for example by site-directed mutagenesis although other techniques may be used. In particular, we have shown that appropriate deletion may be achieved using site-directed mutagenesis for the construction of a recombinant vaccinia virus expressing the env gene of FIV, as will be described in more detail below.

VGR657, a recombinant vaccinia virus expressing the FIV env gene has been described by Rimmelzwaan et al, at the 1st International Conference of feline immunodeficiency virus researches held at U.C. Davis September 4-7, 1991 and is particularly suitable for use according to the present invention, but other vectors or expression systems, including for example other pox viruses (eg. fowl pox virus or canary pox virus), feline rhinotracheitis, adenovirus, baculovirus and yeast, bacterial or mammalian expression systems may of course also be used.

The nucleic acid molecules containing the cleavage site elimination according to the invention as defined above can be expressed in appropriate expression systems well known in the art, to obtain the recombinant FIV polypeptides of the invention.

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The recombinant FIV polypeptides may thus be prepared by expression in a host cell containing a recombinant DNA molecule which comprises a nucleic acid molecule as broadly defined above, operatively linked to an expression control sequence, or a recombinant DNA cloning vehicle or vector containing such a recombinant DNA molecule. Alternatively, the polypeptides may be expressed by direct injection of a naked DNA molecule comprising a nucleotide sequence according to the invention into a host cell. Appropriate recombinant DNA technology and expression techniques are, as mentioned above, well described in the literature for example in Sambrook et al., 1989 (Supra).

The polypeptides so expressed may be fusion polypeptides comprising a FIV polypeptide according to the invention, and an additional polypeptide coded for by the DNA of the recombinant molecule fused thereto. This may for example be β -galactosidase, glutathione-S-transferase, yeast Ty particles, hepatitis core antigen, transmembrane portions of membrane proteins or any other of the polypeptides commonly employed in fusion proteins in the art.

Other aspects of the invention thus include cloning and expression vectors containing DNA comprising nucleotide sequences according to the invention and methods for preparing the nucleic acid molecules of the invention by inserting the said nucleotide sequences into vector nucleic acid, eg. vector DNA. Such expression vectors include appropriate control sequences such as for example translational (eg. start and stop codes) and transcriptional control elements (eg. promoter-operator regions, ribosomal binding sites, termination stop sequences) linked in matching reading frame with the nucleic acid molecules of the invention.

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The invention also includes transformed or transfected prokaryotic or eukaryotic host cells, or transgenic organisms containing a nucleotide sequence according to the invention as defined above, as well as methods for preparing synthetic polypeptides of the invention by culturing host cells containing a nucleic acid molecule as defined above under conditions whereby said polypeptide is expressed and recovering such polypeptide thus produced.

Also included within the scope of the present invention are recombinant nucleic acid molecules which comprise nucleotide sequences according to the invention as defined above together with at least one additional flanking nucleotide sequence, which additional sequence may for example comprise 150 or more bases, preferably no more than 150 bases, more preferably no more than 100 eg. no more than 50 bases. The additional flanking sequences in the nucleic acid molecule according to the invention may be derived from the FIV env gene itself, from other regions of FIV DNA or from heterologous sources, and may be coding or non-coding. In a preferred aspect the flanking sequences may contain one or more restriction sites.

Cloning or expression vectors according to the invention may include plasmids, phage and recombinant viruses, according to techniques well known in the art and may be expressed in a variety of different expression systems cells, including bacterial (eg. E. coli) yeast or mammalian expression systems. To enable appropriate glycosylation of the FIV polypeptides of the invention to take place, expression in eukaryotic, preferably mammalian, systems is preferred. The polypeptides according to the invention may thus be expressed in genetically engineered cell lines eg. cell lines (such as BHK or Hela) transfected with, for example, a virus

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vector, and capable of constitutively expressing the FIV polypeptides according to the invention. Thus, for example, as typical of a suitable viral vector may be mentioned a recombinant vaccinia virus. As a convenient means of expression, a nucleic acid molecule according to this invention may be inserted into a plasmid vector downstream of a vaccinia virus promoter and flanked by vaccinia thymidine kinase (TK) sequences. The resultant recombinant vector is introduced into cells transfected with vaccinia virus. As a result of homologous recombination a TK recombinant vaccinia virus is generated which expresses the polypeptide.

Preferred expression systems according to the present invention include especially mammalian cell systems (eg. BHK cells) but also yeast, Baculovirus and bacterial systems such as E. coli or Salmonella.

Synthetic polypeptides according to the invention may also be prepared by chemical means, such as the well known Merrifield solid phase synthesis procedure.

The nucleic acid sequences and polypeptides according to the invention may be used to prepare vaccine compositions using methods well known in the art of vaccine manufacture.

Traditional vaccine formulations may comprise one or more polypeptides according to the invention together, where appropriate, with one or more suitable adjuvants eg. aluminium hydroxide, muramyl dipeptide, mineral or vegetable oils, novosomes, liposomes, saponins, Quil-A, Q5-21, Matrix or pluronics, in the presence of one or more pharmaceutically acceptable carriers or diluents. Suitable carriers include liquid media such as saline solution appropriate for use as vehicles to introduce the polypeptides into a subject. Additional components

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such as preservatives may be included, as may other antigenic components such as other FIV antigens eg. other FIV proteins and/or polypeptides or other feline virus, eg feline leukaemia virus, antigens, to form a multivalent vaccine.

Immune stimulating complexes (ISCOMS Morein et al., Nature, 308: 457-460, 1984) have recently been found to be particularly effective as adjuvants in vaccine preparations and are particularly attractive in presenting antigens which in native form are membrane bound. In view of their "transmembrane" (TM) portion, ISCOMS are a preferred way of presenting the FIV polypeptide antigens according to the present invention. In particular, we have shown that by eliminating the cleavage site of the FIV envelope protein, incorporation into ISCOMS may be greatly improved. This represents a significant advantage in that ISCOMS are particularly effective in inducing both humoral (eg. virus neutralising antibodies) and cell-mediated immunity (eg. cytotoxic T-cells). Facilitation of ISCOM incorporation in this manner is believed to be due to retention of the highly hydrophobic TM portion of the envelope protein in the constructs of the invention. Accordingly presentation of antigens in this manner represents a particularly favoured and advantageous aspect of this invention.

Such vaccine compositions form a further aspect of the present invention, and the invention can thus be seen also to provide a composition for combatting FIV, preferably a vaccine composition for stimulating an immune response in an animal, preferably a mammal, against FIV, said composition comprising one or more FIV polypeptides comprising an amino acid sequence substantially corresponding to all or a portion of the FIV envelope protein, or an antigenic fragment or

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functionally-equivalent variant thereof, in which the proteolytic cleavage site between the transmembrane and surface polypeptides of the native FIV envelope precursor protein has been eliminated and which comprises at least a portion of a transmembrane polypeptide and at least a portion of a surface polypeptide, together with, optionally, one or more adjuvants, and a pharmaceutically acceptable carrier or diluent, and a method of combatting FIV, preferably a method of stimulating an immune response against FIV in an animal, eg. a mammal, comprising administering to said animal a composition as defined above.

An alternative favourable way of presenting the vaccine according to the invention, is to administer to the animal an expression vector, capable of expressing the polypeptides in question such that the immunogenic polypeptides are expressed *in situ* in the animal. Such "in situ" expression of the antigenic polypeptides has been found to present the immunogen to the immune system of the animal in a particularly favourable manner, and immune responses elicited in this manner are generally significantly improved over those generated using more conventional techniques.

A further preferred aspect of the invention thus includes a composition for combatting FIV, preferably a vaccine composition for stimulating an immune response in an animal against FIV, said composition comprising an expression vector, or host cell having inserted therein a nucleotide sequence according to this invention for stimulation of an immune response directed against polypeptides encoded by the inserted nucleotide sequence, together with one or more pharmaceutically acceptable carrier or diluents.

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Expression vectors suitable for such use are known in the art and include in particular viral vectors, notably pox virus vectors. Vectors worthy of particular mention include the vaccinia virus, fowl pox virus, canary pox virus, feline rhinotracheitis, feline calicivirus, feline panleukopenia adenovirus and bacteria (eg. Salmonella) as described for example by Ada et al., Vaccine 8: 425-437, 1990. Advantageously a fusion protein may be expressed in such a system, where the heterologous protein part (the "carrier") comprises all or a portion of a transmembrane protein (eg. influenza virus HA). When expressed in eukaryotic cells infected with such a recombinant virus, the fusion protein is generally glycosylated and transported to the cell surface through which it protrudes thereby presenting the synthetic polypeptides (or their epitopes) on the outside of the cell surface.

Administration of a composition eg. a vaccine composition according to the invention may take place by any of the conventional routes, eg. orally or parenterally such as by subcutaneous, intramuscular or intradermal injection, optionally at intervals eg. two injections at a 7-42 day interval. Typically a polypeptide is administered in an amount of 1 to 1000 μ g per dose, more preferably 2 to 100 μ g per dose.

The invention will now be discussed in more detail in the following non-limiting Example. In this Example

Figure 1 is a plasmid map showing the construction of plasmid pGR657 used in the construction of recombinant vaccinia viruses (rVV);

Figure 2 shows the results of pulse chase analysis of the generation of FIV proteins after recombinant vaccinia virus (rVV) infection of Hela cells. After infection with rVV's vSC65, vGR657 and vGR657X15, cells

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were labeled metabolically with $^{35}\text{[S]}$ -methionine and $^{35}\text{[S]}$ -cysteine. At 0.2 and 24 hours after labeling FIV env proteins in cell lysates and culture medium were visualized by immune precipitation and PAGE using A) MoAb 6-12-13 recognising FIV TM protein and B) serum from FIV seropositive cat Adam 19;

Figure 3 shows a Western blot analysis of cell lysates of vSC65 (1), vGR657 (2) and vGR657X15 (3) infected BHK cells, using A) MoAb 6-13-12 and B) serum from FIV seropositive cat Adam 19; and

Figure 4 shows a Western blot analysis of ISCOMS prepared with lentil lectin chromatography purified lysates of vGR657 (1) and vGR657X15 (2) infected BHK cells, using A) MoAb 6-13-12 and B) serum from FIV seropositive cat Adam 19.

EXAMPLE 1

Material and Methods

Generation of monoclonal antibodies

BALB/c mice were immunized with ISCOMS in which FIV envelope proteins expressed by recombinant vaccinia virus vGR657 (Rimmelzwaan et al., 1991 supra and as described below) were trapped. For incorporation into ISCOMS, lysates of vGR657-infected RK13 cells were solubilized in phosphate buffered saline (PBS) containing 0.5% NP-40 and 0.5% SDS or 2% MEGA-10 (Boehringer Mannheim, Germany). If the solution remained viscous, it was treated with DNase in the presence of 2 mM MgCl. Subsequently glycoproteins were purified by affinity chromatography using lentil lectin sepharose (Pharmacia, Uppsala, Sweden). Subsequently the purified protein was mixed with the lipids cholesterol and phosphatidyl-ethanolamine (Sigma, St.

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Louis, USA) and Quil A (Spikoside, ISCOTEC AB, Lulea, Sweden) at a ratio of 1:1:5 (w/w) and after ultrasonic treatment for 30 seconds incubated for one hour in the presence of 2% MEGA 10 (Boehringer Mannheim, Germany). Then the mixture was dialyzed against 0.1 M glycine-HCL buffer pH 2.4 for 16 hours at room temperature followed by dialysis against PBS for another 24 hours at 4°C. The thus formed ISCOMS were analysed by electron microscopy, revealing the typical cage-like structure of ISCOMS and by SDS-PAGE followed by Western blot analysis, providing evidence for the incorporation of predominantly transmembrane protein. BALB/c mice were immunized on days 0 and 14 i.p. with approximately 5 µg viral protein incorporated into ISCOM and spleen cells were fused with a mouse myeloma cell line after final intraperitoneal booster injection with a mixture of 5 µg FIV env ISCOM and soluble lentil lectin purified by glycoprotein on day 70.

Enzyme-linked immunosorbent assay for the detection of FIV env-specific MAb's

Microtiter plates (Costar RIA/EIA, high binding) were coated with recombinant FIV glycoproteins obtained from lysates of vGR657-infected RK13 cells in 0.1 M NaAc buffer pH 5.5 for 16 hours at room temperature. Then the antigen was aspirated and the wells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing the plates with water containing 0.05% Tween 80 the plates were blocked with PBS containing 0.05% Tween 80 and 10% foetal calf serum (FCS). Undiluted culture supernatants were transferred to the wells and incubated for one hour at 37°C. After washing, a horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Fc) antibody preparation (Cappel, Cooper Biomedical, Malvern, USA) was added and incubated for another hour at 37°C. The plates were washed and 100 µl

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volumes of substrate solution (0.1 mg/ ml tetramethyl benzidin [TMB] and 0.003% H_2O_2 in 0.1 M NaAc buffer pH 5.5) were added to each well. After incubation for 10 min. at 20°C 100 μ l volumes of 2M H_2SO_4 were added to stop the color reaction. The absorbance at 450 nm (A450) was read in a Titertek Multiscan (Titertek, Flow Laboratories).

MoAb 6-12-13, a monoclonal antibody selected for these studies provided to be specific for the transmembrane (TM) protein of FIV as demonstrated by showing its reactivity with FIV env proteins in RIPA (see below and Fig. 1a) and immunostaining of Western blots (Fig. 2a and 3a).

Construction of recombinant vaccinia virus

The FIV env gene (2.6 Kb) encoding the envelope precursor protein was amplified by PCR using bone marrow derived DNA, obtained from a FIV-infected cat as source of FIV proviral DNA (Siebelink et al., J. Virol, 66: 1091-1097, 1992). The oligonucleotides in the PCR amplification were synthesized on an Applied Biosystems (Foster City, CA, USA) DNA synthesizer. Sequences of the oligonucleotides are as follows:

GGCAGTTGCAATCTACATTATC and GCAACAATAAGAATGGCAG (prime from the 5' end), CTCAGCACAGTATCTCCC and GACATACCTTCCTCAAAGGG (prime from the 3' end) and were derived from the nucleotide sequence of the petaluma strain of FIV (Talbot et al., 1989, supra). The amplified product was subsequently cloned into the Hinc II site of the E. coli cloning vector pBluescript II SK(+) (Stratagene, La Jolla, CA. USA). Insertion of the FIV env gene was confirmed by restriction endonuclease analysis, followed by DNA hybridization using gel purified env gene as labeled probe (ECL, Amersham, UK) and by shotgun sequencing of the FIV env gene. The FIV

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env gene was excised from the Bluescript construct using restriction enzymes Xho 1 and Sma 1, and cloned into the Sal 1 and Sma 1 sites of plasmid pSC65 as shown in Figure 1. pSC65 contains vaccinia virus TK sequences flanking the cloning site, a synthetic early/late vaccinia promoter and the lac Z gene under control of the vaccinia 7.5K promoter. The resulting plasmid was designated pGR657 (fig. 1). Constructed plasmids were screened by restriction endonuclease analysis and DNA hybridization with gel purified env gene as labelled probe (ECL, Amersham, UK), and were grown by standard procedures (Sambrook et al., 1989, supra).

For the construction of a recombinant vaccinia virus (rVV) that expresses the gp160 precursor protein of FIV in which the cleavage site between the OM and TM protein has been deleted, we used the plasmid pSC65. The procedure to create recombinant vaccinia virus vGR657X15, containing a deletion in the nucleotide sequence at 1822-1833 positions coding for the cleavage site RRKR was as follows: 3 µg of the pBluescript construct containing the env gene (pBS/env) (Rimmelzwaan et al., 1991, supra) was used as template DNA for amplification by PCR using primers 5' - del (5' - GGAAGTCATGGAATATAAACCTGCAGCTATTCATGTTATGTTGGC-3'; positions 1800-1856 in which positions 1822-1833 were deleted and 3' Bgl II (5'-GGGGGTAGATCTTTTGTGGTTATACC-3'; positions 2204-2227), to obtain fragment 5'-del/3'-Bgl II. Using primer pair 3'-del (reverse complementary to 5'-del) and 5'-NSI (5'-GACCTTATTATGCATTTCAATATGACAAAAGCTG-3'; position 1534-1567), fragment 5'-NSI 1/3'-del was obtained. To obtain the 5'-NSI 1/3'-Bgl II fragment containing the deletion, a PCR was performed with the 5'-NSI 1 and 3'-Bgl II primers and 500 ng of the fragments 5'-NSI 1/3'-del and 5'-del/3'-Bgl II as template. The PCR-derived 5'-NSI1/3'-Bgl II fragment was purified by preparative gel

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electrophoresis, digested with NSI I and Bgl II and cloned into NSI I/Bgl II digested pGR657. The resulting plasmid was designated pGR657X15. The presence of the deletion was confirmed by determination of the nucleotide sequence in this region using the dideoxynucleotide chain termination sequence reaction.

Subsequently recombinant viruses were generated as previously described (Mackett et al., 1985), by homologous recombination with vaccinia virus (WR strain), which originally was obtained from the American type culture collection. Recombinant virus plaques were visualized by their blue color as a result of the co-expression of the lac Z gene and overlay with 5 bromo-4 chloro-3 indolyl-BD-galactopyranoside (X-gal) (Chakrabarti et al., 1985, Molecular Cellular Biology 5: 3403-3409). The virus was plaque purified three times and large stocks of vGR657 were grown in HeLa cells.

Protein expression

For radioimmunoprecipitation TK-143 B cells were infected with 30 pfu of recombinant vaccinia virus per cell and metabolically labeled for 18 hours with [³⁵S]methionine (ECL, Amersham, UK). Cells were lysed in PBS containing a 0.5% NP-40 and proteins were immunoprecipitated as previously described using a serum from a cat infected with FIV (Chlody et al., AIDS Research and Retroviruses, 3(2): 165-176, 1987). The proteins were analyzed by SDS-PAGE (Laemli 1970).

For Western blotting, infected cells were lysed 24 hours post-infection and proteins were separated by SDS-PAGE (Laemli 1970) and electrically transferred to nitrocellulose (Towbin). After blocking the nitrocellulose sheets with PBS containing 0.1% Tween 80 and 0.5% milk-powder, they were incubated with either

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anti-FIV cat sera or monoclonal antibody.

Subsequently the nitrocellulose was incubated with biotin-labeled mouse anti-cat IgG (Clone CT-21, Sigma, St. Louis, USA), or a goat anti-mouse IgG antibody preparation, respectively. Between each incubation the nitrocellulose was washed three times with PBS containing 0.5% Tween 80. Finally the blots were incubated with alkaline phosphatase (AP) complexed extravidin (Sigma, St. Louis, USA) followed by color reaction using BCIP/NTB as substrate.

Analysis of radiolabeled FIV glycoproteins

The FIV proteins expressed by rVV vGR657 and vGR657X15 were analysed by pulse-labeling in a radio immunoprecipitation assay (RIPA) and Western blot analysis. For RIPA, Hela cells were infected with recombinant vaccinia viruses VGR657, VGR657X15 or VSC65 (control rVV made with pSC6S) at a multiplicity of infection (m.o.i) of 30 pfu per cell for 4 hours. For pulse-chase analysis, infected cells were incubated for 30 minutes in methionine- and cysteine- free medium (RPMI) prior to pulse-labeling for 30 minutes with 200 μ Ci of [32 S] methionine/cysteine (specific activity >1000 Ci/mmol) per ml. The pulse-labeling was followed by a chase period of 0.2, or 24 hours. Culture supernatant as well as cell lysate was analysed for presence of FIV specific proteins. Culture supernatant was centrifuged to remove cells. Cells were washed with phosphate buffered saline (PBS) and lysed in lysis buffer (100 mM Tris-HCl, pH 8.0; 100 mM NaCl; 0.5% Triton X-100 and 0.2 mM PMSF). The cell lysates were clarified by centrifugation. FIV-specific proteins were immune precipitated from both culture supernatants and cell lysates with polyclonal anti-FIV antibody (serum from the naturally infected cat Adam 19) (Siebelink *et al.*, 1992 *Supra*) and a monoclonal antibody (MoAb) 6-13-12 specific for FIV transmembrane

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protein (see above).

To avoid non-specific binding, culture supernatant and cell lysate were incubated with serum from an FIV sero-negative cat and protein-A sepharose beads. After 1 hour incubation at 4°C the protein-A sepharose beads were removed by centrifugation. IgG from serum from an FIV sero positive cat was coupled to protein-A sepharose beads. After 1 hour the beads were washed twice with triton buffer (0.3 M NaCl; 50 mM Tris-HCl, pH 7.4 and 0.1% triton X-100®).

For immune precipitation pre-absorbed culture supernatants and cell lysates were incubated with protein-A sepharose beads coupled with IgG from an FIV seropositive cat or with monoclonal antibody MoAb 6-13-12 for 2 hours at 4°C. The antigen-antibody complex bound to the protein-A sepharose beads were collected by centrifugation and washed twice with triton buffer and once with DOC buffer (0.3 M NaCl; 50 mM Tris-HCl, pH 7.4; 0.1% SDS and 0.1% Na-deoxycholate). The final pellet was resuspended in SDS-PAGE loading buffer and heated for 10 minutes at 100°C. Sepharose beads were then removed by centrifugation. The samples were analyzed by SDS polyacrylamide gel electrophoresis (PAGE).

RESULTS

Analysis of FIV glycoproteins produced by recombinant vaccinia virus

The results of labeling recombinant vaccinia viruses VSC65, VGR657 and VGR657X15 infected Hela cells followed by immune precipitation and PAGE are shown in Figure 2. The pattern of the proteins which are produced by the cells which are infected with VGR657 or VGR657X15 are compared with those of cells infected with VSC65. In

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the cell lysate of cells infected with VGR657 two FIV specific proteins with MW of 150,000 and 130,000 were observed directly after pulse labeling. After a chase period of 2 hours the 150 kd protein had disappeared and only one protein of MW 130,000 was seen. After a chase period of 24 hours, a smear of FIV specific protein with a mean molecular weight of 32 kd and a 95 kd protein were visualised in addition to the 130 kd protein. In the culture supernatant of the cultures infected with VGR657 no FIV specific proteins could be detected in the cultures which were not chased or were chased for 2 hours, whereas a protein with a MW of about 95,000 was detected after a chase period of 24 hours (Fig. 2B). These results show that VGR657 infected cells produce a FIV specific precursor envelope protein with a MW of 150,000 which is rapidly processed to a protein with a MW of 130,000. This protein is subsequently cleaved into a protein of approximately MW 95,000 which is released in the culture supernatant and most likely represents the surface protein (SU) of FIV, and a protein of molecular weight approximately MW 32,000 which most likely represents the trans membrane protein (TM) of FIV and is cell-associated. Thus the processing of FIV glycoproteins expressed by rVV looks similar to the processing of FIV glycoproteins in persistently infected Crandell feline kidney (CrFK) cells as described by Stephens, et al., (1991), J. Virol. 65: 1114-1123).

In the cell lysate of cells infected with rVV VGR657X15 also two FIV specific proteins with MW of 150,000 and 130,000 could be detected directly after pulse labeling whereas a strong band with MW of 130,000 and a weak band with MW 150,000 could be seen after a chase period of 2 hours. Only one FIV specific protein with MW 130,000 was observed after a chase period of 24 hours. In the culture supernatant of the cultures infected with VGR657X15 no FIV specific proteins could be detected

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(Fig. 2B). These data suggest that in cells infected with vGR657X15, an FIV precursor envelope protein with an apparent molecular weight of 150 kd was synthesized and that this protein was processed into a 130 kd protein that is not cleaved like the native protein. Immunoprecipitation studies with MoAb 6-12-13 using the same samples confirmed this suggestion. With this MoAb, the 150, 130 and 32 kd proteins, but not the 95 kd protein, were immunoprecipitated from lysates of cells infected with rVV vGR657 (Fig. 2A). From cells infected with rVV vGR657X15, proteins with molecular weight of 150 kd and 130 kd were precipitated. No 32 kd protein was precipitated from these lysates, indicating that the 130 kd protein, expressed by vGR657X15, is not cleaved. The molecular weights observed for the full length precursor, SU and TM proteins fall within the range of the published values (Miyazawa et al., Supra). Any differences are assumed to be due to differences in glycosylation.

When lysates of rVV infected BHK cells were tested for the presence of FIV proteins by Western blot analysis, the 130 kd protein could be visualized in vGR657X15 infected cells but not in vSC65 or vGR657 infected cells. In vGR657 infected cells the TM protein could be detected but not in vSC65 or vGR657X15 infected cells (Fig. 3), which confirms the findings of the pulse labeling experiment.

Incorporation studies with lentil lectin purified extracts from BHK cells infected with the respective rVV's showed that ISCOM prepared from purified extracts of rVVGR657X15 infected cells (as described above) incorporated predominantly the whole uncleaved 130 kd protein, in addition to minor amounts of TM protein (32 kd protein), as shown in Western blot analysis following polyacrylamide gel electrophoresis using MoAb 6-13-12 (Fig. 4A) or serum from seropositive cat Adam 19 (Fig.

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4B). We speculated that the presence of the 32 kd protein was the result of a putative secondary cleavage site and that this protein was found now, as a result of the concentration of the hydrophobic proteins during the process of ISCOM formation. In contrast, ISCOM prepared from similarly purified extracts of rVV vGR657 infected cells, incorporated no 130 kd protein, but did incorporate the TM protein and a 64 kd protein, which was speculated to represent a dimer of the TM protein (Fig. 4). The 130 kd protein incorporated in the ISCOM matrix is of particular interest as immunogen since it may be expected to contain the majority of the T and B cells epitopes of the native envelope proteins.

The modified 130 kd envelope protein has been shown to sufficiently stable for ISCOM incorporation and is therefore proposed as a suitable candidate vaccine antigen for FIV.

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Claims

1. A synthetic FIV polypeptide comprising an amino acid sequence substantially corresponding to all or a portion of the FIV envelope protein, or an antigenic fragment or functionally-equivalent variant thereof, in which the proteolytic cleavage site between the transmembrane and surface polypeptides of the native FIV envelope precursor protein has been eliminated and which comprises at least a portion of a transmembrane polypeptide and at least a portion of a surface polypeptide.
2. A synthetic FIV polypeptide as claimed in claim 1 for use in combatting FIV.
3. A synthetic FIV polypeptide as claimed in claim 1 or claim 2 wherein said proteolytic cleavage site has been deleted.
4. A synthetic FIV polypeptide as claimed in any one of claims 1 to 3, in the form of a fusion protein comprising an additional polypeptide fused to said amino acid sequence, or which is coupled to a carrier protein or polypeptide.
5. Use of a synthetic FIV polypeptide as defined in any one of claims 1 to 3 in the preparation of a composition for combatting FIV.
6. A nucleic acid molecule comprising a nucleotide sequence substantially corresponding to all or a portion of the FIV env gene encoding the FIV envelope protein, or a sequence which is degenerate or substantially homologous with or which hybridises with any such aforesaid sequence, wherein in said nucleotide sequence the sequence encoding the proteolytic cleavage site

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between the transmembrane and surface polypeptides of the native FIV envelope protein has been eliminated, and wherein said nucleotide sequence encodes at least a portion of a transmembrane polypeptide and at least a portion of a surface polypeptide.

7. A nucleic acid molecule as claimed in claim 6 for use in preparing FIV-combatting compositions.

8. A nucleic acid molecule as claimed in claim 5 or claim 6 further comprising at least one additional flanking nucleotide sequence, in addition to said FIV env-encoding sequence.

9. Use of a nucleic acid molecule as defined in any one of claims 6 to 8 in the preparation of a composition for combatting FIV.

10. An expression or cloning vector comprising a nucleic acid molecule as defined in any one of claims 6 to 8.

11. An expression vector as claimed in claim 10 which is a recombinant vaccinia virus.

12. A host cell or transgenic organism containing a nucleic acid molecule as defined in any one of claims 6 to 8, or an expression vector as claimed in claim 10 or claim 11.

13. A host cell as claimed in claim 12 which is a mammalian cell.

14. A method for preparing a synthetic FIV polypeptide as defined in any one of claims 1 to 4, which comprises culturing host cells as defined in any one of claims 12 or 13, under conditions whereby said polypeptide is

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expressed, and recovering said polypeptide thus produced.

15. A composition for combatting FIV comprising one or more synthetic FIV polypeptides as defined in any one of claims 1 to 4, or an expression vector or host cell having inserted therein a nucleic acid molecule as defined in any one of claims 6 to 8, for stimulation of an immune response directed against polypeptides, encoded by the inserted nucleic acid molecule, together with, optionally, one or more adjuvants, and a pharmaceutically acceptable carrier or diluent.

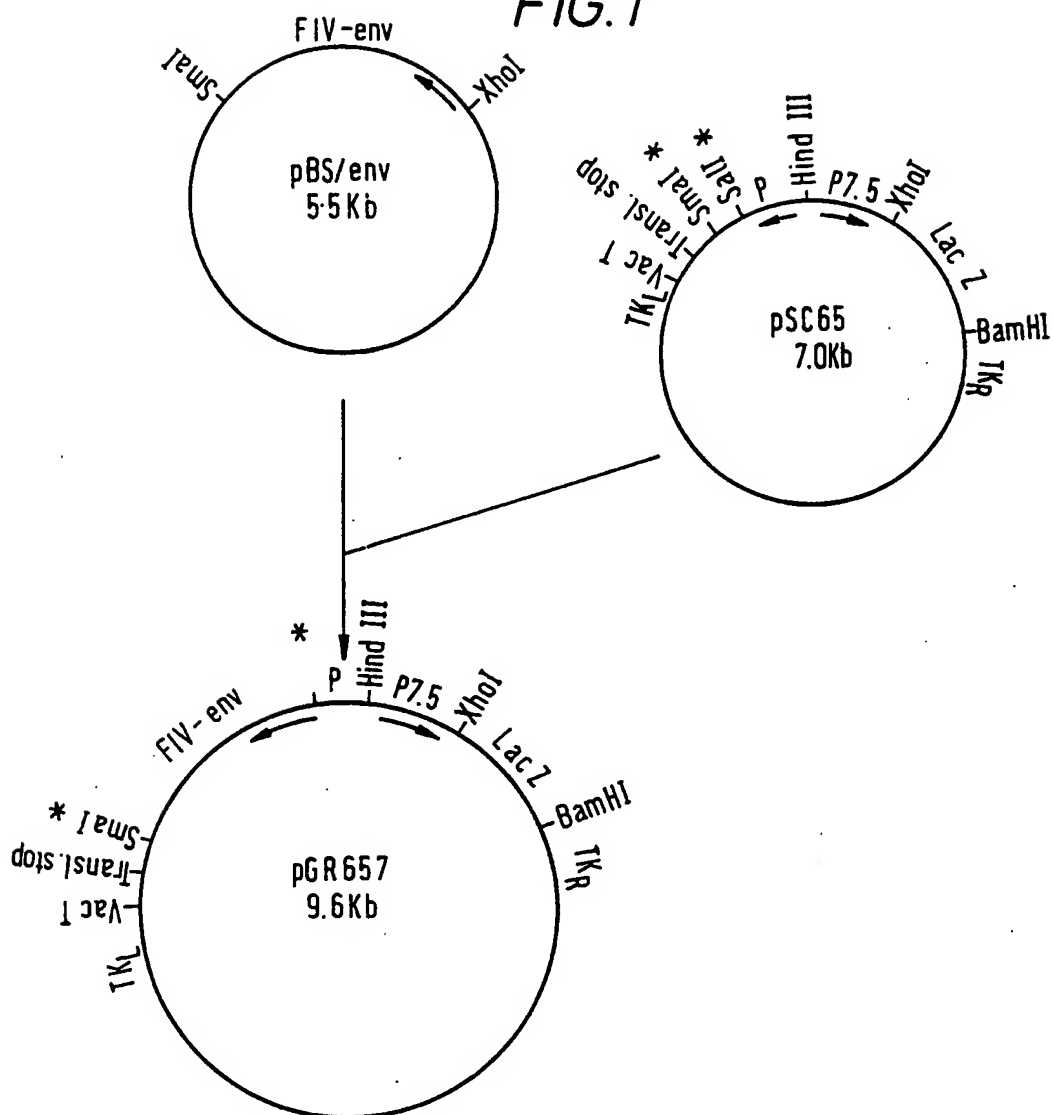
16. A vaccine composition as claimed in claim 15, comprising one or synthetic polypeptides as defined in any one of claims 1 to 4, incorporated into ISCOMS.

17. A vaccine composition as claimed in claim 15 or claim 16, additionally comprising one or more other antigenic FIV proteins and/or peptides.

18. A method of combatting FIV, comprising administering to an FIV-infected animal, a composition as claimed in any one of claims 15 to 17.

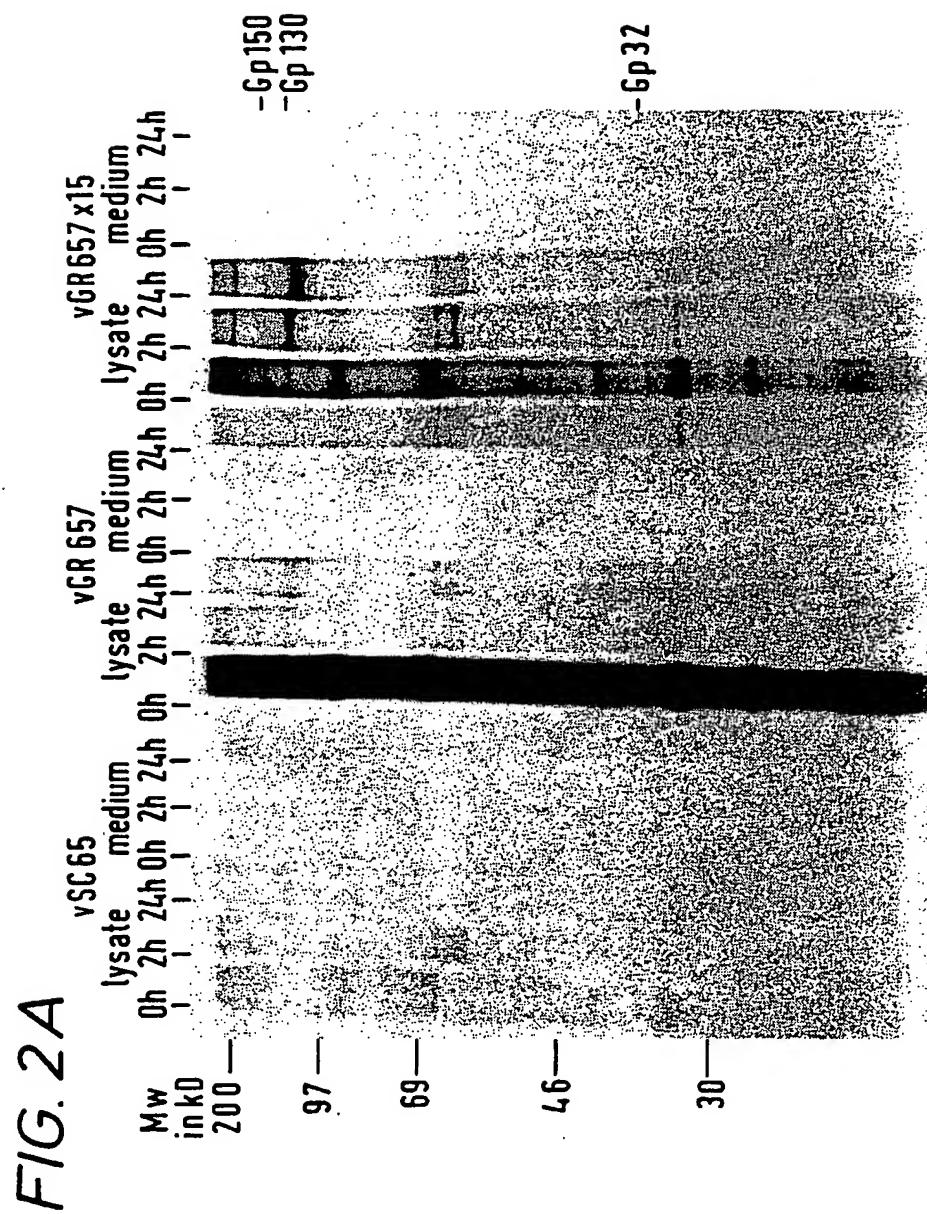
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FIG. 1



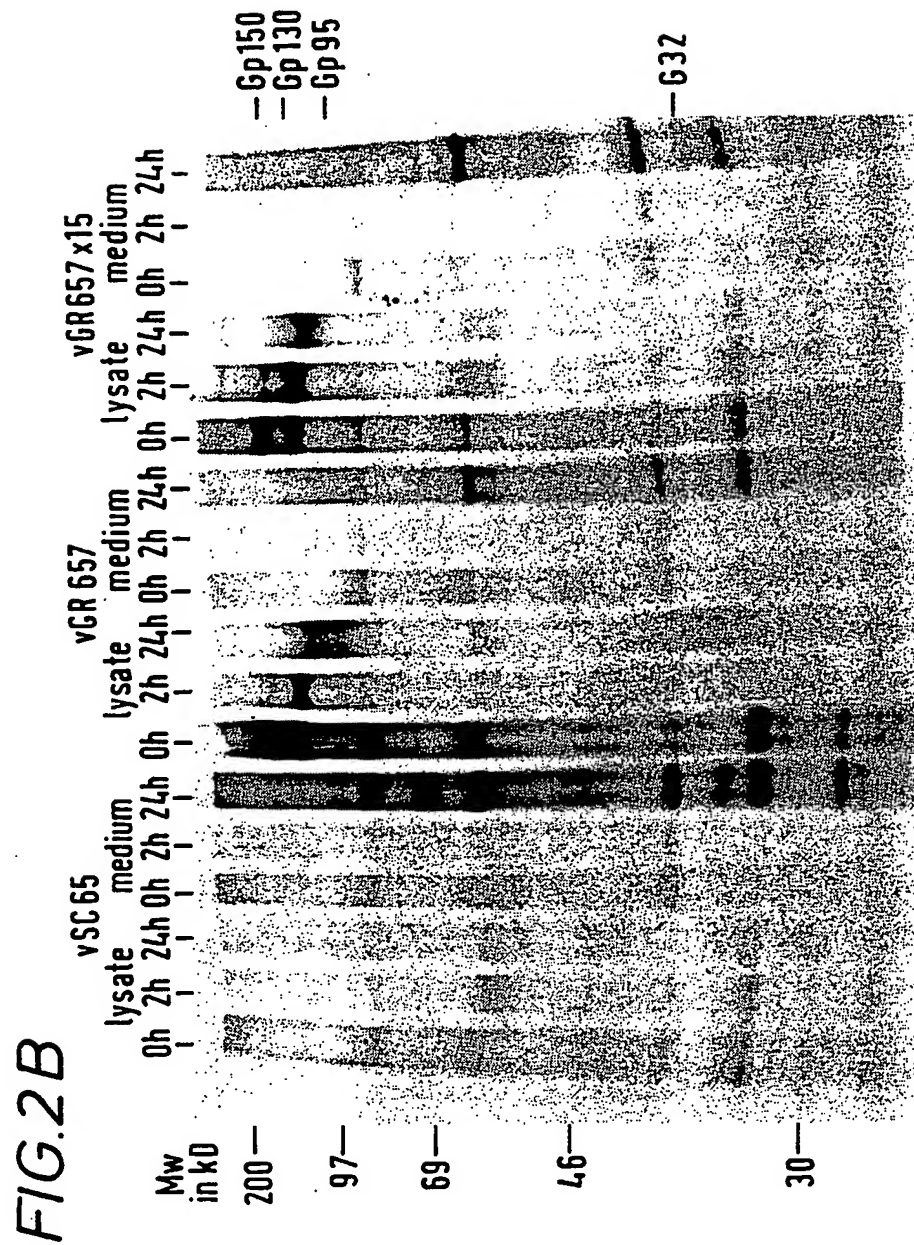
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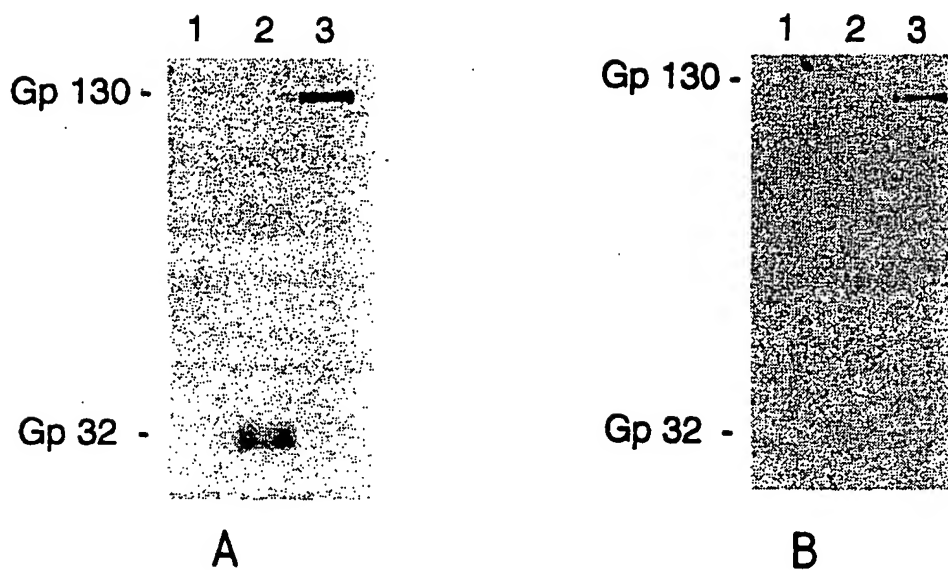


FIG. 3

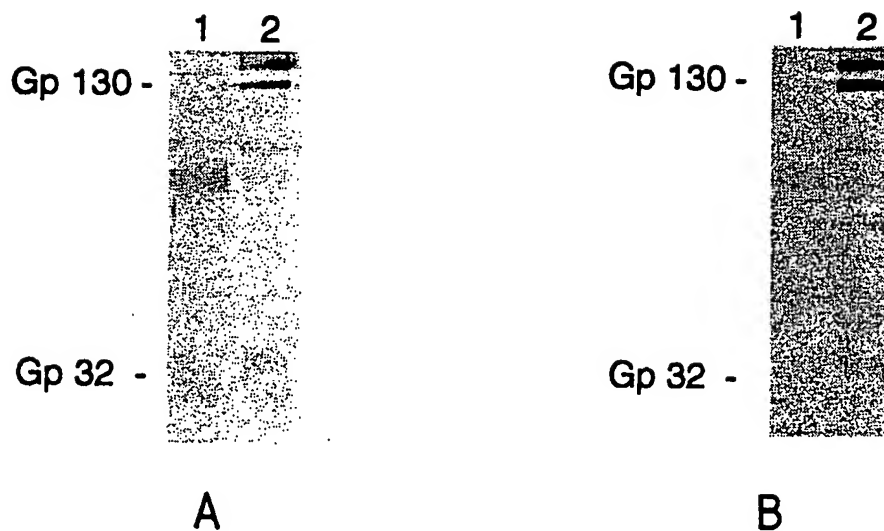


FIG. 4

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/01861

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/49; A61K39/21; C07K13/00; C12N15/86 C12N5/10		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; A61K ; C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	JOURNAL OF VIROLOGY vol. 66, no. 2, February 1992, pages 1091 - 1097 K.H. SIEBELINK ET AL. 'Isolation and partial characterization of infectious molecular clones of feline immunodeficiency virus directly obtained from bone marrow DNA of a naturally infected cat' cited in the application see the whole document ---	1-17
Y	EP,A,0 245 136 (TRANSGENE S.A. & INSTITUT PASTEUR) 11 November 1987 see example 6 ---	1-17
	--- -/--	
¹⁰ Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
24 SEPTEMBER 1993	01 -10- 1993	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	CUPIDO M.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 335 635 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 4 October 1989 see the whole document ---	1-17
A	WO,A,9 209 632 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 11 June 1992 see page 4, line 6 - page 5, line 17; figure 1 ---	1-17
O,P, X	File AIDSLINE Abstract 00071711 Int. Conf AIDS July 19-24 1992, 8(2)pA42 Abstract PoA 2237:G.F. Rimmelzwaan et al. Construction of recombinant vaccinia viruses expressing the feline immunodeficiency virus (FIV) env-gene -----	1-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 93/01861

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 18 is directed to a method of treatment of the animal body, the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9301861
SA 77248

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

24/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0245136	11-11-87	FR-A- 2596771	09-10-87
		FR-A, B 2606029	06-05-88
		AU-B- 604696	03-01-91
		AU-A- 7234987	09-11-87
		WO-A- 8706260	22-10-87
		JP-T- 1500161	26-01-89
		US-A- 5169763	08-12-92

EP-A-0335635	04-10-89	AU-A- 3170089	28-09-89
		JP-A- 2009372	12-01-90

WO-A-9209632	11-06-92	FR-A- 2669338	22-05-92
